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Apical-to-basolateral transepithelial transport of Ochratoxin A by two subtypes of Madin-Darby canine kidney cells

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Abstract

In this study we investigated the transepithelial transport of Ochratoxin A (OTA), a potent nephrotoxin, across monolayers of two collecting duct-derived cells clones (Madin-Darby canine kidney cells (MDCK)-C7 and MDCK-C11 cells, resembling principal and intercalated cells, respectively) either from the apical to the basolateral side or vice versa. We cultured cells on permeable supports and compared the transport rates of OTA, *p*-aminohippuric acid (PAH) and fluorescein-labelled inulin. Monolayers of both cell clones translocated OTA from the apical to the basolateral side but not in the opposite direction. Transport rate across MDCK-C11 cell monolayers was 2.9-fold the transport rate across MDCK-C7 cell monolayers. OTA transport was temperature-dependent being reduced from 77.5 pmol/cm^2 per h to 10.1 pmol/cm^2 per h in MDCK-C11 and from 27.0 pmol/cm^2 per h to 7.6 pmol/cm^2 per h in MDCK-C7 cells when temperature was decreased from 37°C to 4°C. In both cell clones, the dipeptides carnosine and glycylsarcosine but not the amino acids glycine or phenylalanine had an inhibitory effect on OTA transport. In both cell clones, transepithelial transport of OTA was dependent on the apical pH (pK_a of OTA = 7.1). In an environment mimicking the transepithelial in vivo pH gradient to some extent with more acidic pH on the apical side than on the basolateral side, transport was 4-fold higher in both cell clones as compared to conditions when pH was 7.4 in both bath solutions. In the absence of a pH gradient, transport rates were similar to that at 4°C. Apical uptake of [^{3}H]OTA was inhibited by carnosine and by glycylsarcosine and the uptake of [^{3}H]carnosine was inhibited by OTA. Our results indicate that OTA is transported across the apical membrane of MDCK cells by both non-ionic diffusion and by a H^+-dipeptide cotransporter. Thus, reabsorption of OTA in the collecting duct contributes to the observed long half life of OTA in the mammalian body.

Keywords: Ochratoxin A; Transport; Madin-Darby canine kidney cell; Dipeptide cotransport; Kidney; Collecting duct

1. Introduction

Ochratoxin A is a potent nephrotoxic agent produced by several fungi of *Aspergillus* and *Penicillium* species. Via contaminated animal feed and human food it also threatens human health [1–4]. OTA is bound to albumin and to so far unknown small proteins in the blood to more than 99% [5,6] and it remains in the body for long times (up to 510 h in apes [7]). Porcine Nephropathy and perhaps also Balkan Endemic Nephropathy are visible damages caused by OTA in man and animals [8–12]. In the kidney proximal parts of the nephron [13–15] and also postproximal parts are affected by OTA where it

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blocks plasma membrane anion conductance and deranges pH homeostasis [16,17].

In the kidney, proton-coupled transporters of oligopeptides have been described and cloned [18–20]. They transport also dipeptide-mimicking molecules like β-lactam antibiotics [21,22]. OTA consists of a 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3*R*-methylisocoumarin linked over its carboxy group to L-β-phenylalanine [23] and is thus similar to dipeptides. Therefore, peptide carriers might play a role in apical OTA transport leading to its reabsorption from the collecting duct. Because reabsorption of OTA in these postproximal sections could contribute to OTA's long half life in the body, we used two cell clones, MDCK-C7 and MDCK-C11 cells, resembling principal and intercalated cells of the collecting duct which have been isolated recently in our laboratory from the MDCK cell wildtype [24] to investigate transport of OTA across renal epithelial cell monolayers.

2. Materials and methods

2.1. Cell culture

Madin-Darby canine kidney cells (MDCK-C7 and MDCK-C11 cells [24], passage 65–80) were cultured as described [17] in MEM medium, pH 7.4, supplemented with Earl's salts, non-essential amino acids, 10% fetal calf serum (Biochrom KG, 12213 Berlin, FRG), and 26 mmol/l $NaHCO_3$ at 37°C and 5% CO_2. For transport studies, cells were seeded on permeable supports (membrane filters, 4.9 cm^2, Falcon, Becton-Dickinson, 69006 Heidelberg, FRG) and incubated for three days in the above mentioned medium until they had reached confluence. Furthermore, confluence of the cell monolayer was confirmed by measuring the transepithelial electrical resistance with a voltohmmeter (EVON, WPI, Sarasota, USA, MDCK-C7 cells $794.3 \pm 129.2\ \Omega \times cm^2$, $n = 32$ and MDCK-C11 cells $319.1 \pm 33.2\ \Omega \times cm^2$, $n = 32$, remaining stable during the experiment).

2.2. Transepithelial transport studies

For transport studies, the medium was exchanged against Hepes-Ringer buffer (in mmol/l: Hepes (*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethane sulfonic acid) 10, NaCl 122.5, KCl 5.4, $MgCl_2$ 0.8, $CaCl_2$ 1.2, NaH_2PO_4 1, glucose 5.5). Medium volumes were 1.3 ml apical and 2.5 ml basolateral. The pH was individually adjusted for each experiment, usually, in the basolateral bath pH was 7.4, apical either 7.4 or 6.0. OTA (usually 5 μmol/l), PAH (5 mmol/l) and fluorescein isothiocyanate (FITC)-Inulin (100 mg/ml) were added either to the apical or the basolateral bath solution as indicated for each experiment. The experiments were performed either at 37°C or at 4°C in room atmosphere. If not stated otherwise, the OTA-free bath solution was exchanged completely against fresh bath solution every 30 min for the first 2 h. The last sample was taken after additional 2 h of incubation.

OTA and PAH content in the removed bath solution was determined by HPLC as described below. FITC-Inulin was determined by fluorometric detection at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. The relative amount of OTA transported that exceeds the relative amount of transported PAH represents transcellular transport under the assumption that MDCK cells do not express the organic anion carrier.

2.3. Determination of intracellular OTA content

After incubation, cells were rapidly washed six times with ice-cold phosphate buffered saline (PBS-buffer, in mmol/l: NaCl 141, KCl 4, $MgCl_2$ 1, $CaCl_2$ 1, NaH_2PO_4 0.4, Na_2HPO_4 1.6, glucose 5, pH 7.4). Cells were opened by adding 0.1% Triton X-100 to the buffer, and after 15 min incubation at room temperature lysed cells were collected in a centrifuge cap. After centrifugation at $8000 \times g$ (10 min) the protein in the supernatant was precipitated with three volumes ethanol. This ethanol treatment completely removes OTA from the protein and leads to 99% recovery of OTA in the supernatant as shown previously in our laboratory [25]. After final centrifugation ($8000 \times g$, 10 min), the supernatant was vacuum dried, and the OTA content was determined by HPLC as described below.

2.4. HPLC

OTA was determined under isocratic conditions using a reverse phase C-18 column (Nova Pak C18,

150 × 3.9 mm, Millipore Waters GmbH, 65731 Eschborn, FRG) and subsequent fluorometric detection (RF-551, Shimadzu, Japan) at an excitation wavelength of 330 nm and an emission wavelength of 460 nm. The flow rate was 1 ml/min. According to [3] the mobile phase consisted of acetonitrile (45%, Promochem, 46469 Wesel, FRG), deionized water (53%, Seral, 56235 Ransbach-Baumbach, FRG) and glacial acetic acid (2%, Merck, 64271 Darmstadt, FRG). Under these conditions the solvent peak appeared 1.3 min, the peak of OTα, a metabolite of OTA, 2.8 min, and the peak of OTA 4.5 min after injection.

PAH was determined under isocratic conditions using a reverse phase C-18 column (Spherisorb-5ODS, 25 × 0.46 cm, particle size 5 μm, Kontron, 85386 Eching, FRG) and subsequent photometric detection at 235 nm (Kontron UV spectrophotometer). The flow rate was 1.3 ml/min, the mobile phase consisted of 20% acetonitrile, deionized water and 0.8% glacial acetic acid. PAH absorbance could be detected 3.5 min after injection.

2.5. Apical uptake experiments and determination of intracellular radioactivity

Experiments were done three days after seeding in 24-well dishes with an established confluent cell monolayer. The medium was exchanged against Hepes-Ringer buffer pH 6.0. The concentration of [^{3}H]OTA was 84 nmol/l (137 TBq/mol), the concentration of methoxy [^{14}C]inulin, which served as extracellular control substance, was 0.73 μmol/l (2.11 TBq/mol). After the experiment, cells were washed immediatly with ice-cold PBS buffer. Cells were detached by 60 minutes incubation with 0.1% trypsin solution in PBS containing 0.02% EDTA and collected in a scintillation tube. 4 ml scintillation fluid (Packard, Meriden, USA) were added and radioactivity was determined in a Packard Liquid Scintillation Analyzer (Packard, Meriden, USA).

2.6. Enzyme assay and protein- and cell volume determination

Lactate dehydrogenase activity in the buffer or in Triton-X 100-lysed cells were measured according to [26] at room temperature in a photometer. Protein was determined according to [27]. Cell volume was calculated as follows: The average number of cells on one filter (4.9 cm^2) was $2.5 \cdot 10^6$ cells ($n = 7$). Cell volume was determined to 2.2 pl/cell by a cell counter and analysis system (Coulter counter, Schärfe System, 72770 Reutlingen, FRG). This gives a total cell volume of 5.5 μl/filter and 1.61 μl per well (1.43 cm^2).

2.7. Chemicals

Crystalline OTA was purchased from Sigma (St. Louis, USA). [^{3}H]OTA was purchased from Moravek Biochemicals, Brea, USA. Methoxy [^{14}C]inulin was purchased from DuPont NEN, Bad Homberg, FRG. Carnosine, L-[β-alanine-3-^{3}H], was purchased from American Radiolabeled Chemicals, St. Louis, USA. All other chemicals were obtained from Merck (64271 Darmstadt, FRG).

2.8. Statistics

All measurements are given as mean values ± S.E. The significance of difference was determined by the unpaired Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Viability of cells after OTA treatment

For initial control, we incubated MDCK-C7 and MDCK-C11 cells with 5 μmol/l OTA for 4 h in Hepes-Ringer buffer pH 6.0 and measured the lactate dehydrogenase (LDH) activity in the buffer and inside the cells as an indicator of the extent of cytolysis. Only 0.81% of the total LDH-activity could be detected in the medium of MDCK-C11 cells after 4 h incubation (0.82% in MDCK-C7 cells). Control cells incubated without OTA showed 0.96% and 0.79% LDH-activity in the media of MDCK-C11 and MDCK-C7 cells, respectively ($n = 3$). This shows that during the 4 h experiment the viability of the cells is not impaired by OTA.

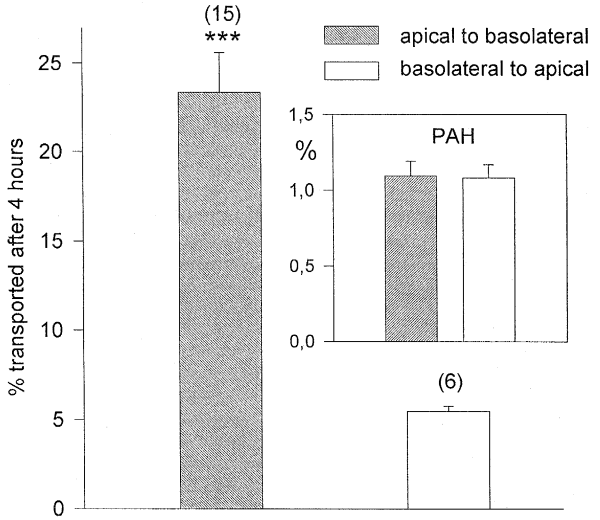

Fig. 1. Transepithelial transport of ochratoxin A (OTA) and *p*-aminohippurate (PAH) from the apical to the basolateral side and vice versa in MDCK-C11 cells. Transport was studied at pH 6.0 apically and pH 7.4 basolaterally (apical to basolateral) or at pH 7.4 apically and pH 6.0 basolaterally (basolateral to apical) at 37°C for 4 h. Inset shows transport of PAH ($n = 6$). 5 μmol/l OTA and 5 mmol/l PAH were added apically or basolaterally, and the respective opposite bath solution was exchanged every 30 min. The amounts of transported substances in the respective opposite bath solution were summed up. Values are given as percent of apically or basolaterally added amount of substances. The number of filters is shown in brackets. *** indicates a $P < 0.001$ compared to basolateral to apical transport.

3.2. Transepithelial OTA transport in MDCK-C11 cells

3.2.1. Reabsorptive transepithelial OTA transport in MDCK-C11 cells

After 4 h incubation 309 ± 29.8 pmol/cm^2 OTA were transported from the apical (pH 6.0) to the basolateral (pH 7.4) bath ($n = 15$). This corresponds to 23.4% of OTA added to the apical bath. However, if OTA was added to the basolateral (pH 6.0) side, only 72.1 ± 4.0 pmol/cm^2 OTA were transported to the apical (pH 7.4) side during 4 h incubation, corresponding to 5.4% of added OTA ($n = 12$, Fig. 1). Thus, transcellular transport of OTA occurs only from the apical to the basolateral compartment. In contrast, PAH transport was equal in both directions (1.09% and 1.08% of added PAH, respectively). The directional OTA transport also excludes a transport via tight junctions of the cell. However, if OTA was added simultaneously in equal concentrations (5 μmol/l) to both sides of the epithelium, no accumulation of OTA in one of both bath solutions could be detected after 4 h of incubation at 37°C (not shown). Thus, the cell layer was not able to establish a transepithelial OTA concentration gradient.

3.2.2. pH dependence of transepithelial OTA transport in MDCK-C11 cells

OTA transport was clearly dependent on the apical pH. When pH was raised from 6.0 to 7.4 on the apical side, only 65.1 ± 19.3 pmol/cm^2 per 4 h were transported to the basolateral bath (pH 7.4) compared to 309 ± 29.8 pmol/cm^2 per 4 h at pH 6.0 ($n = 9$). However, PAH and FITC-Inulin transport were independent of pH (Fig. 2).

3.2.3. Temperature dependence of transepithelial OTA transport in MDCK-C11 cells

309 ± 29.8 pmol/cm^2 OTA, corresponding to 23.4% of OTA added to the apical bath solution were

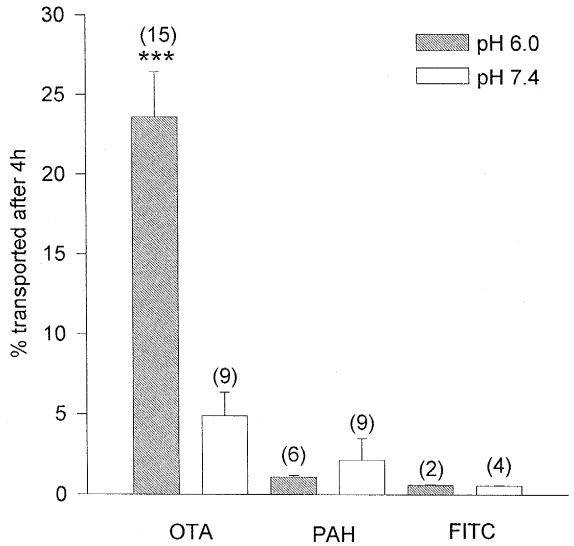

Fig. 2. pH-dependent transepithelial transport of ochratoxin A (OTA), *p*-aminohippurate (PAH) and FITC-Inulin (FITC) in MDCK-C11 cells. 5 μmol/l OTA, 5 mmol/l PAH, and 100 mg/l FITC-Inulin were added apically. Transport from the apical to the basolateral bath was studied at 37°C at an apical pH of 6.0 or pH 7.4 for 4 h. Basolateral pH was 7.4 in both experiments. Basolateral bath solution was exchanged every 30 min and the amounts of the respective transported substances in the basolateral bath were summed up. Values are given as percent of apically added chemicals. *** indicates a $P < 0.001$ compared to pH 7.4. The number of filters is shown in brackets.

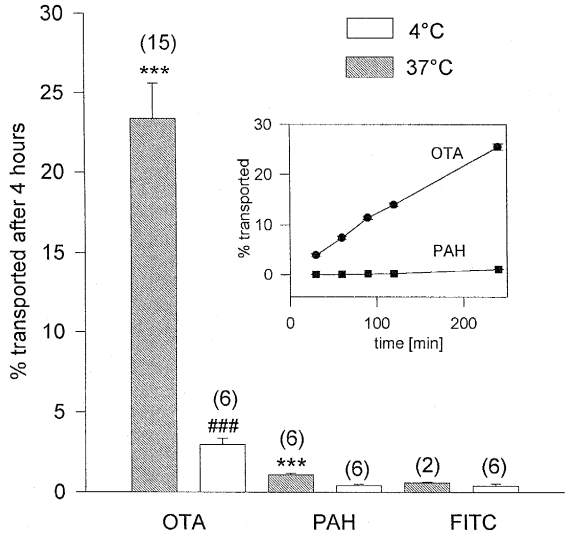

Fig. 3. Temperature dependence of cumulative transepithelial transport of ochratoxin A (OTA), *p*-aminohippurate (PAH) and FITC-Inulin (FITC) from the apical to the basolateral bath solution in MDCK-C11 cells after 4 h incubation. Apical pH was 6.0 and basolateral pH 7.4. 5 μmol/l OTA, 5 mmol/l PAH, and 100 mg/l FITC-Inulin were added apically, and the basolateral bath solution was exchanged every 30 min. The amounts of transported substances in each sample of one filter were summed up. Values are given as percent of apically added substances. *** indicates a $P < 0.001$ compared to 4°C, ### indicates a $P < 0.001$ compared to the PAH transport, number of filters in brackets. Inset shows time course of OTA (●) and PAH transport (■).

transported to the basolateral side within 4 h at 37°C and pH 6.0 ($n = 15$). This corresponds to 86.5% of the OTA-flux measured on uncolonized control filters during 4 h incubation and demonstrates the high capacity of the transport systems involved. Only 39.4 $\pm$ 5.5 pmol/cm^2 per 4 h of OTA corresponding to 2.97% of the added amount were transported at a temperature of 4°C ($n = 6$, Fig. 3). Thus, at 4°C OTA transport was reduced to 12.7% as compared to that at 37°C. This shows that transepithelial OTA transport needs cellular activity and is not only a simple diffusion process. However, even at 37°C the PAH- and the FITC-Inulin content in the basolateral bath solution was virtually nil although PAH has only half the molecular weight of OTA. But the relative amount of transported OTA at 4°C is still higher than the relative amount of PAH transported at 37°C. The low amount of PAH and FITC-Inulin transported again proves the tightness of the cell monolayer during the experiment.

3.3. Transepithelial OTA transport in MDCK-C7 cells

3.3.1. Reabsorptive, pH- and temperature-dependent transepithelial OTA transport in MDCK-C7 cells

MDCK-C7 cell monolayers also transported OTA in a pH- and temperature-dependent manner. Furthermore, only apical to basolateral transport could be detected as already shown for the MDCK-C11 cells. When OTA was added to the basolateral side instead of the apical side, only 0.4% of OTA added to the basolateral side were translocated to the apical side after 4 h incubation (which represents 54.8 fmol/cm^2, Fig. 4). Transport from the apical to the basolateral bath under optimal conditions (pH 6.0 apically and 37°C) was 108.1 pmol/cm^2 after 4 h incubation corresponding to 8.2% of added OTA ($n = 15$) and was less than that observed for MDCK-C11 cells. At low temperature (4°C), only 2.3% of added OTA were transported after 4 h (29.8 pmol/cm^2, $n = 9$).

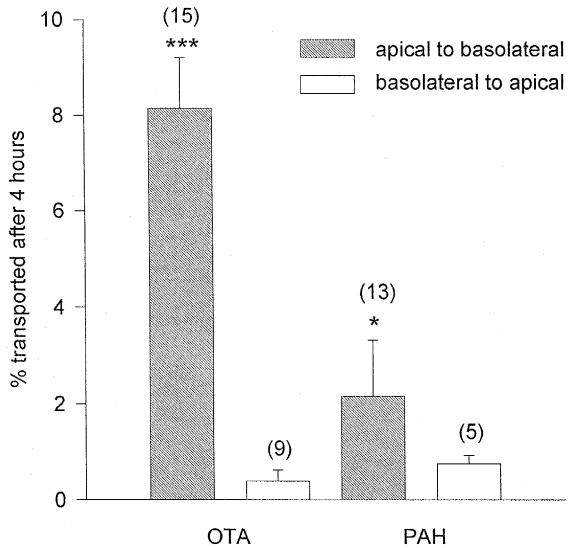

Fig. 4. Transepithelial transport of ochratoxin A (OTA) and *p*-aminohippurate (PAH) from the apical to the basolateral bath and vice versa in MDCK-C7 cells. Transport was studied at pH 6.0 apically and pH 7.4 basolaterally at 37°C for 4 h. OTA (5 μmol/l) and PAH (5 mmol/l) were added apically or basolaterally and the respective opposite bath solution was exchanged every 30 min. Transported amounts of OTA or PAH, respectively, in the opposite bath were summed up. Values are given as percent of apically or basolaterally added substances. The number of filters is shown in brackets. *** indicates a $P < 0.001$ compared to basolateral to apical OTA transport, * indicates a $P < 0.05$ compared to basolateral to apical PAH transport.

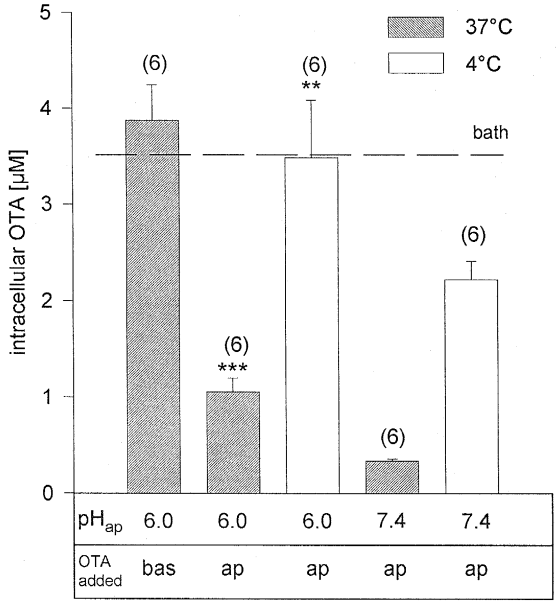

Fig. 5. Intracellular ochratoxin A (OTA) concentration (μmol/l cell volume) after 4 h incubation in MDCK-C11 cells. The pH of the apical bath was either 6.0 or 7.4. 3.5 μmol/l OTA (horizontal line) were added to the apical (ap) or basolateral (bas) bath and after 4 h the cells were washed and opened with Triton X-100 and OTA determined. bas = basolateral, ap = apical. Number of samples in brackets. *** indicates a $P < 0.001$ compared to pH 7.4 and ** indicates a $P < 0.005$ compared to pH 7.4.

3.4. Intracellular OTA concentration

In MDCK-C7 and MDCK-C11 cells, intracellular OTA concentration (μmol/l cell volume) after 4 h incubation was dependent on temperature, pH and on the side to which OTA was added. If OTA was added apically, equimolarity of intracellular OTA with that in the apical bath solution could be detected only at 4°C (3.5 μmol/l), whereas at 37°C the intracellular OTA concentration was significantly lower than that in the apical bath (1.06 μmol/l, Fig. 5). However, if OTA was present on the basolateral side, also at 37°C intracellular OTA concentration was equal to the basolateral OTA concentration (3.5 μmol/l) although virtually no OTA could be detected in the apical bath solution under these conditions as described above. Additionally, at an apical pH of 6.0, more OTA could be detected inside the cells as compared to pH 7.4.

3.5. Inhibition of transepithelial OTA transport by apically added dipeptides in MDCK-C7 and MDCK-C11 cells

Because of the phenylalanine moiety and the peptide bond of OTA, and the pH dependence of transport, a H^+-dipeptide cotransporter might be involved

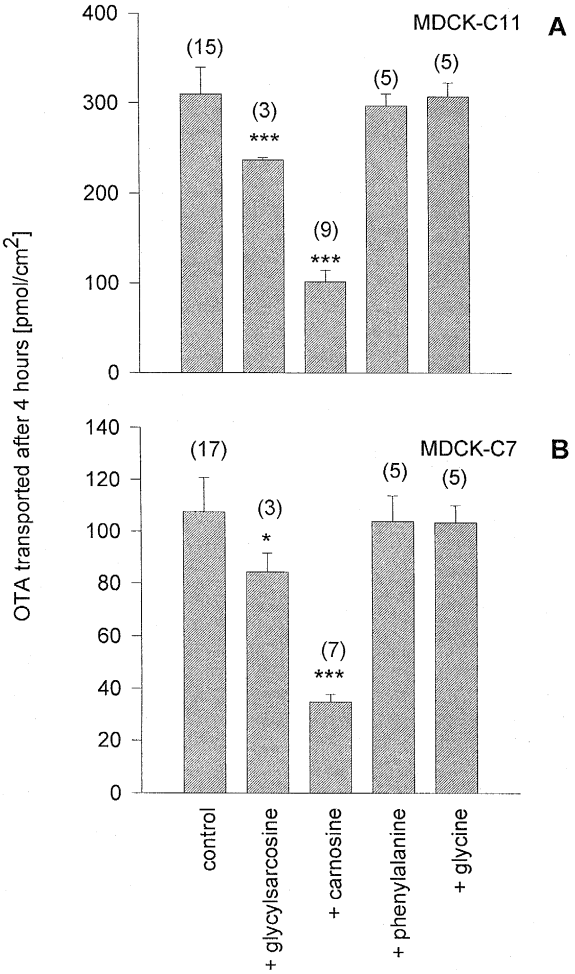

Fig. 6. Effect of dipeptides and amino acids on transepithelial OTA transport. (A) MDCK-C11 cells. (B) MDCK-C7 cells. Note the different scales of the ordinates. Transport was studied at pH 6.0 apically and pH 7.4 basolaterally and at 37°C for 4 h. Basolateral bath solution was exchanged every 30 min. Dipeptides (carnosine 5 mmol/l, glycylsarcosine 50 mmol/l) or amino acids (5 mmol/l) and OTA (5 μmol/l) were added apically and the amount of transported OTA was determined in the basolateral bath and summed up. *** indicates a $P < 0.001$ compared to control and * indicates a $P < 0.05$ compared to control.

in the apical uptake of OTA into the cell [28]. To prove this, we studied transport of OTA in the presence of the dipeptides glycylsarcosine and carnosine at pH 6.0 apically. In both cell clones studied, carnosine and glycylsarcosine inhibited OTA transport significantly (Fig. 6). Carnosine was a more potent inhibitor than glycylsarcosine. In the presence of 5 mmol/l carnosine transport was reduced from 309.9 pmol/cm^2 per 4 h to 103.25 pmol/cm^2 per 4 h in MDCK-C11 cells ($n = 9$) and from 108.1 pmol/cm^2 per 4 h to 34.9 pmol/cm^2 per 4 h in MDCK-C7 cells ($n = 7$) whereas in the presence of 50 mmol/l glycylsarcosine transport was reduced to 237.1 pmol/cm^2 per 4 h in MDCK-C11 cells and to 84.2 pmol/cm^2 per 4 h in MDCK-C7 cells. The single amino acids phenylalanine or glycine (5 mmol/l) had no effect on OTA transport (Fig. 6).

3.6. Apical uptake of [^{3}H]OTA by MDCK-C11 cells

The time course of [^{3}H]OTA uptake showed that the intracellular [^{3}H]OTA content reached its maximum after 30 min incubation at pH 6.0 and 37°C (15 min: 937.9 ± 11.8 pmol/ml cell volume; 30 min: 1932 ± 20.8 pmol/ml cell volume and 60 min: 2072 ± 31.4 pmol/ml cell volume, $n = 4$). OTA uptake was linear in the concentration range tested (up to 250 μmol/l OTA). After 30 min incubation at 37°C and pH 6.0, uptake of [^{3}H]OTA was inhibited by 5 mmol/l carnosine from 64.1 ± 0.8 pmol/min per ml cell volume to 36.6 ± 1.4 pmol/min per ml cell volume ($n = 4$; $P < 0.01$) and by 5 mmol/l glycylsarcosine to 50.5 ± 2.7 pmol/min per ml cell volume ($n = 4$; $P < 0.05$) corresponding to a decrease to 57.6% and 79.3% of maximal uptake, respectively. The uptake of ^{3}H-labelled carnosine (19.7 ± 0.46 fmol/min per ml cell volume, $n = 4$; 5 nmol/l solution, 1480 TBq/mol) into MDCK-C11 cells was inhibited by 100 μmol/l OTA (to 14.1 ± 1.51 fmol/min per ml cell volume, corresponding to 71.5% of maximal uptake; $n = 4$, $P < 0.001$) and by 50 mmol/l glycylsarcosine or carnosine (to 9.4 ± 0.92 and 9.5 ± 0.36 fmol/min per ml cell volume, corresponding to 29.5% and 30.0% of maximal uptake, respectively, $n = 4$; $P < 0.001$) after 30 min incubation. This shows that OTA interacts with a dipeptide uptake system in the apical cell membrane.

4. Discussion

The toxic effects of OTA are at least in part enhanced by its unusual long presence in the mammalian body [7]. Besides its strong binding to plasma proteins such as albumin [5,6], which prevents an effective filtration in the kidney, a reabsorptive uptake of OTA along the nephron may additionally increase its persistence in the body and thus intensify its toxicity. For this reason, and as earlier studies of our laboratory showed that the function of the collecting duct is highly affected by OTA [16,17] we studied the transport of OTA in cells derived from the collecting duct (MDCK-C7 and MDCK-C11 resembling principal and intercalated cells of the collecting duct, respectively [24]), in order to uncover such transport capabilities. We found a clear pH- and temperature-dependent transepithelial transport of OTA which occurs only from the apical to the basolateral side but not vice versa proving that collecting duct cells are able to 'reabsorb' OTA.

In both cell clones, transepithelial OTA transport was temperature-dependent. At 4°C, the transport rate in MDCK-C11 and MDCK-C7 cells was significantly reduced to 12.6% and to 27.6% resp. of the transport rate observed at 37°C. However, at 4°C intracellular OTA concentration was increased as compared to 37°C and even equalled the apical OTA concentration whereas at 37°C, significantly less OTA was detected inside the cell. This suggests that an additional carrier-mediated, temperature-dependent and thus active process is located at the basolateral side of the cell. At 37°C the carrier extrudes OTA across the basolateral membrane keeping the intracellular concentration low. In contrast, at 4°C and thus with low cellular activity OTA enters the cell at the apical side passively but is prevented from leaving the cell across the basolateral membrane.

The dependence of the cellular OTA concentration on temperature and the side of OTA exposure suggests that the apical membrane acts as a valve which allows the entry but not the exit of any OTA across the apical membrane. The only way for OTA to leave the cell via the basolateral membrane is a temperature-dependent process and is the rate limiting step. This is in good accordance to findings in the human intestinal Caco-2 cell line where the export of glycylsarcosine across the basolateral membrane limits its

transepithelial transport [29]. Unfortunately, only little is known about basolateral exit of oligopeptides [30]. It has been suggested that, in the intestine, basolateral export is a proton-peptide symport but different from the proton-dipeptide cotransport at the apical membrane [31,32]. However, in MDCK cells net transport did not occur if the basolateral bath has the same OTA concentration as the apical bath. This shows that basolateral OTA transport is facilitated diffusion and thus is not able to establish a concentration gradient. In the blood, however, albumin and other plasma constituents [5,6] can act as a binding sink for OTA so that in vivo a constant drag for OTA out of the cell into the blood exists.

The phenylalanine moiety of OTA leads to an interaction of OTA with phenylalanine-handling proteins such as phenylalanyl-tRNA synthetase or phenylalanine hydroxylase [33,34]. Proton-coupled oligopeptide cotransporter not only transport dipeptides (e.g. carnosine) but also similar molecules such as β-lactam antibiotics [22,35,36]. As OTA consists of an isocoumarin ring linked to phenylalanine by a peptide bond it is possible that OTA is a substrate of an apical H^+-peptide cotransporter [28,30,37]. The presence of a H^+-dipeptide cotransport system with a K_m of 1.3 mM in MDCK-cells has been described recently [38]. In that study, uptake of dipeptides was pH-dependent as was the transport of OTA in the present study. Our results which show that (i) OTA transport through MDCK-C11 and MDCK-C7 cell monolayers was not inhibited by single amino acids but by the dipeptides carnosine or glycylsarcosine; (ii) that both dipeptides inhibit [^{3}H]OTA entry into the cell across the apical membrane and (iii) that OTA inhibits [^{3}H]carnosine uptake point to the involvement of a H^+-dipeptide cotransporter in the OTA transport route. OTA uptake was not saturated in the micromolar range which is not a surprise considering the K_m of 1.3 mM of the dipeptide cotransport system in MDCK cells [38]. We did not use higher OTA concentrations to avoid unspecific and toxic effects of OTA as described in [39]. The reason why carnosine inhibits uptake and transepithelial transport more effectively than glycylsarcosine remains unclear. But as transepithelial transport is studied, different actions of the dipeptides on either intracellular proteins or basolateral export is conceivable.

At pH 7.4, OTA is transported at rates even lower than those found in the presence of dipeptides at pH 6.0 and similar to those at 4°C. This indicates that besides the remaining activity of transporter, transport of OTA is in addition a pH-dependent diffusion (simple or facilitated) process on the apical membrane. This is supported by the present finding that the intracellular OTA concentration is high even after incubation at 4°C. OTA with a pK_a of 7.1 is less charged and thus better membrane diffusible at pH 6.0 (92% of total OTA uncharged) than at pH 7.4 (33% uncharged). Once inside the cell, OTA is trapped by the more alkaline conditions which recharge the OTA molecule (50% charged) so that it is less able to rediffuse across the membranes. As expected, the transport of PAH (pK_a 3.8) and of FITC-Inulin did not show this pH dependence. Their transport rates did not differ significantly at the two pH values.

To which extent intracellular proteins interact with this transport process is not known. Previous studies in our laboratory demonstrated OTA binding to proteins in both MDCK cell clones and in OK-cells [25]. Therefore, a trapping effect of intracellular protein cannot be excluded. Yet, metabolism of OTA by these cells is very unlikely because we could not detect any known OTA metabolite [40–42] in the HPLC analysis.

Transport of OTA by simple leakyness of the monolayer or a transport via cell junctions can be excluded because of its apical-to-basolateral unidirectionality and because of its temperature and pH dependence. Additionally, in the case of leakyness the rate of PAH transport expressed as percent of total amount should equal OTA transport. Also cell death can be excluded because no increase in LDH activity was detectable in the culture medium.

In conclusion our results show that OTA is transported across the apical membrane of MDCK cells by non-ionic diffusion and possibly by a proton-coupled dipeptide cotransporter. OTA leaves the cell by a temperature-dependent process at the basolateral side. Additionally, a reabsorption of OTA in the collecting duct in vivo was demonstrated recently in our laboratory [43] so that the reabsorptive capability of the collecting duct leads to a fatal cycling of OTA in the body. Any OTA that is filtered can be reabsorbed in the collecting duct and thus would contribute to the

unusual long half live of OTA in the mammalian body thus increasing the risk of severe diseases caused by this mycotoxin.

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